ORIGINAL RESEARCH COMMUNICATION

Phenolic acid intake, delivered via moderate Champagne wine consumption, improves spatial working memory via the modulation of hippocampal and cortical protein expression/activation

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ABSTRACT

Aims: Whilst much data exists for the effects of flavonoid-rich foods on spatial memory in rodents, there are no such data for foods/beverages predominantly containing hydroxycinnamates and phenolic acids. To address this we investigated the effects of moderate Champagne wine intake, which is rich in these components, on spatial memory and related mechanisms relative to alcohol- and energy-matched controls. **Results:** In contrast to the iso-caloric and alcohol matched controls, supplementation with champagne wine (1.78 ml/kg BW, alcohol 12.5 % vol.) for 6 weeks led to an improvement in spatial working memory in aged rodents. Targeted protein arrays indicated that these behavioral effects were paralleled by the differential expression of a number of hippocampal and cortical proteins (relative to the iso-caloric control group), including those involved in signal transduction, neuroplasticity, apoptosis and cell cycle regulation. Western immunoblotting confirmed the differential modulation of BDNF, CREB, p38, dystrophin, CNPase, mTOR, Bcl-xL in response to champagne supplementation compared to the control drink, and the modulation of mTOR, Bcl-xL and CREB in response to alcohol supplementation. **Innovation:** Our data suggest that smaller phenolics such as gallic acid, protocatechuic acid, tyrosol, caftaric acid and caffeic acid, in addition to flavonoids, are capable of exerting improvements in spatial memory via the modulation in hippocampal signalling and protein expression. **Conclusion:** Changes in spatial working memory induced by Champagne supplementation are linked to the effects of absorbed phenolics on cytoskeletal proteins, neurotrophin expression and the effects of alcohol on the regulation of apoptotic events in hippocampus and cortex.

INTRODUCTION

Declines in both cognitive and motor performance due to structural- and activity-related changes in neuronal and glial function are known to occur during normal aging (15). Recently, flavonoid containing foods/beverages have received much attention with regards to their neuroprotective effects (51), including a potential to protect neurons against neurotoxin-induced injury (42, 66), suppress neuroinflammation (59), and promote memory and learning (31, 44, 47, 62). In addition to this, numerous epidemiological studies have reported that a low to moderate intake of wine (1-2 glasses per day), which is also rich in flavonoids, may reduce the risk of coronary heart disease, ischemic stroke, dementia and cognitive impairment (26, 28, 29, 39). As such, there is an interest in the potential of regular, moderate wine consumption to counteract normal brain ageing and in improving memory and learning, through their potential to deliver relatively high amounts of flavonoids (8, 10). However, although red wines contain high levels of flavonoids and other phenolics relative to white wines (33), Champagne wine is relatively rich in phenolic compounds (68) such as hydroxybenzoic acids, hydroxycinnamic acids (and their tartaric derivative esters), phenolic alcohols and phenolic aldehydes (6). The increased levels of phenolics in Champagne wine compared to other white wines, derive predominantly from the two red grapes, Pinot Noir and Pinot Meunier, which are used in its production along with the white grape Chardonnay (9). As such, it is hypothesized that Champagne may deliver significant quantities of phenolics, which may in turn be capable of driving vascular and neuronal effects capable of mediating changes in cognitive performance.

Previously, Champagne wine consumption has been observed to improving acute vascular function (64), in a similar manner to that of red wine (7, 12). Furthermore, tyrosol, caffeic acid, and gallic acid, phenolic compounds found at relatively high concentrations in Champagne, have been shown to potently inhibit peroxynitrite-induced cellular injury at physiologically relevant concentrations (0.1 to 10 μM) (68), whilst nanomolar levels of tyrosol, caffeic acid and *p*-coumaric acid protect cortical neurons against 5-S-cysteinyl-dopamine induced injury (63). Indeed, the levels of protection induced by these phenolics was equal to, if not greater than, that observed for similar concentration of the flavonoids, (+)-catechin, (-)-epicatechin and quercetin (63). The hydroxycinnamate, caffeic acid, has also been shown to be neuroprotective, counteracting inflammatory injury induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) by

decreasing the production of a number of inflammatory cytokines, down-regulating the expression of iNOS, COX-2 and glial fibrillary acidic protein and lowering the production of NO and PGE_2 (58). In addition, caffeic acid phenethyl ester may protect cerebellar granule neurons against glutamate-induced neuronal death via inhibition of p38 phosphorylation and caspase-3 activation (71) and significantly prevents hypoxic-ischaemic-induced neonatal rat brain damage in the cortex, hippocampus and thalamus (72).

Despite these data, the impact of such phenolics on neuronal function/activity has received little attention relative to flavonoids, in particular in animal and human trials, and there is a lack of understanding with regards to their mechanisms of action in the brain *in vivo*. In particular, there is no data relating to the consumption of small phenolics and hydroxycinnamtes, such as those present in Champagne wine on memory and neuro-cognitive performance. To address this, we have investigated the potential effects of moderate, medium-term Champagne wine intake on age-related deficits in spatial working memory and motor performance and the potential mechanisms of action in the hippocampus and cortex.

RESULTS

Analysis of phenolic constituents in Champagne wine

The HPLC analysis of the phenolic extract obtained from Champagne wine allowed us to identify and quantify the major constituents present (Figure 1). The chromatographic profile of the organic extract at 280nm (Figure 1A) and 320nm (Figure 1B) highlights the major phenolic components in the Champagne extract utilized: gallic acid (peak 1), protocatechuic acid (peak 2) tyrosol (peak 3), caftaric acid (peak 4) and caffeic acid (peak 5); whereas the overlapping standard runs of the flavonoids catechin (std 6), epicatechin (std 7) and resveratrol (std 8) indicate the absence of these compounds in the extract in detectable levels. The major phenolics identified (Figure 1C) were quantified in order to precisely characterize intake on supplementation of the Champagne intervention (Fig 1D).

Food intake and animal weight: Animals were fed daily with Champagne, an iso-caloric control or an iso-caloric alcohol containing control (Figure 1D). There were no significant differences in food intake among the three groups throughout the study period (iso-caloric control: 20.81 g/day; alcohol control: 20.42 g/day; Champagne group: 20.36 g; $p>0.05$). At baseline, control, alcohol and Champagne group animals weighed 478.11 (±18.23), 484.63 (±48.06) and 455.15 (±36.97) g, respectively, and there was no significant weight change throughout the intervention period in any of the groups (average weights at week 6: 480.70 ± 15.75, 490.80 ± 50.43 and 462.19 ± 31.20 g, respectively; $P>0.05$).

Spatial working memory: At baseline the animals performed at an average choice accuracy of 4.25 (±0.27) and there was no significant difference in choice accuracy between the three experimental groups (Figure 2). At 6 weeks there was a reduction in the choice accuracy of both iso-caloric control group (3.50 ±0.27) and alcohol group (4.00 ±0.38) and an increase in the choice accuracy of the Champagne group (5.29 ±0.18). A two-way ANOVA with repeated measures indicated that there were no significant changes with respect to time ($F_{(1,20)}=0.022$, MSE=0.026, $p=0.884$), or between treatments ($F_{(2,20)}=1.863$, MSE=2.734, $p=0.181$), but there was a trend to significance with regards to the interaction between treatment and time

($F_{(2,20)}$=2.955, MSE=3.536, p=0.075). Bonferroni post-hoc analysis indicated that this trend to significance was predominantly driven by a significant difference in choice accuracy between the iso-caloric control group and the Champagne group at 6 weeks (P<0.01).

Motor performance: A two-way ANOVA with repeated measures indicated that there was no significant affect of the interventions on distance covered by the animals on beam walking tests (time: $F_{(1,19)}$=1.101, MSE=0.621, p=0.307; treatment: $F_{(2,19)}$=0.855, MSE=4.058, p=0.441; interaction: $F_{(2,19)}$=1.080, MSE=0.609, p=0.360) (Figure 2A). Similarly, latency time (Figure 2B) was not significantly affected by control or Champagne intervention for 6 weeks (time: $F_{(1,19)}$=1.703, MSE=33.783, p=0.208; treatment: $F_{(2,19)}$=0.441, MSE=36.414, p=0.650; interaction: $F_{(2,19)}$=0.293, MSE=5.814, p=0.749). In agreement with this, there were no significant effects of any of the treatments on calculated walking speed (Figure 2C; time: $F_{(1,19)}$=1.742, MSE=41.637, p=0.203; treatments ($F_{(2,19)}$=1.155, MSE=52.296, p=0.336; interaction: $F_{(2,19)}$=0.690, MSE=16.500, p=0.514). These data suggest that neither alcohol nor Champagne intervention had an influence on motor performance.

Protein expression array: 40 hippocampal (Figure 4) and 31 cortical (Figure 5) proteins related to signal transduction, neuroplasticity, apoptosis and cell cycle regulation were significantly modulated (p<0.05) following 6 weeks of Champagne intake relative to the iso-caloric control. In both hippocampus and cortex, Champagne intervention modulated a range of proteins involved in the MAPK signaling and the epidermal growth factor receptor signaling cascade, such as p38 and phospho-Raf. In addition, in the cortex Champagne down-regulated the expression of some calcium-activated protein kinases (PKC a, PKC b and PKC g) and phospholipases C and A2. Champagne intervention increased the expression of the myelin-associated enzyme CNPase in the hippocampus, and a range of neuroplasticity-related proteins in both hippocampus and cortex (Dystrophin, plakoglobin, tryptophane hydroxylase, spectrin), whereas it down-regulated NGFR p75 and NMDAR 2a receptors in cortex only. Finally, Champagne supplementation also led to the modulation of apoptotic proteins (i.e. caspases and Bcl family proteins) and cell cycle/nuclear

proteins, including that of the anti-apoptotic proteins Bcl-xL and Bcl-x, and cyclin A and Cdc27 in both hippocampus and cortex. Probe protein array analysis provided several new candidate proteins which were selected based on expression levels and role in neuronal plasticity (p38, CNPase, dystrophin and Bcl-xL), along with previously identified proteins BDNF, pro-BDNF, CREB, mTOR, Erk, JNK, Akt, Arc, and PKA C-α absent in the array, for further evaluation and/or confirmation by western immunoblotting. A complete list of the proteins analyzed with the protein array is presented as supplementary material (Supplementary Fig. S1: hippocampus and Fig. S2: cortex) Supplementary Data are available online at www.liebertonline.com/ars).

Immunoblotting: In comparison to the isocaloric and alcohol control interventions, Champagne intervention led to significant increases in the hippocampal levels of phospho-p38, pCREB (Ser^{133}) and BDNF ($p<0.001$; $p<0.05$ and $p<0.001$ respectively) (Figure 6A). In addition, there was a significant positive correlation between hippocampal BDNF levels in individual animal performance on spatial memory tasks ($R=0.613$, $P<0.01$) (Figure 6B) and between hippocampal phospho-p38 levels and spatial performance ($R=0.681$, $P<0.01$). Similarly, Champagne intervention led to the increased expression of dystrophin and CNPase ($p<0.05$ and $p<0.01$ respectively) (Figure 7), with significant positive correlation existing between spatial memory task performance and hippocampal levels of dystrophin ($R=0.74$, $P<0.05$) and CNPase ($R=0.529$, $P<0.05$).

In contrast, mTOR phosphorylation and Bcl-xL were altered by both the Champagne and alcohol treatments (Figure 8). Hippocampal levels of Bcl-xL were found to be significantly reduced in animals supplemented with both Champagne ($p<0.001$) and alcohol ($p<0.01$) compared to control, although to a greater extent in the Champagne group ($p<0.001$). mTOR activation on the other hand was significantly higher in both Champagne ($p<0.001$) and alcohol-supplemented animals ($p<0.05$) compared to the iso-caloric control. Champagne-induced hippocampal changes in p38, CNPase, Dystrophin and Bcl-xL levels in the hippocampus were in agreement with that observed using antibody arrays (Figure 4). There were no significant alterations in the activation state, or expression of, Akt, Arc, Erk, JNK, PKA C-α and pro-BDNF in the hippocampus in response to either alcohol or Champagne intervention (Table 1).

With regards to protein expression in the cortex, the effect on mTOR was opposite to that observed in the hippocampus, with both alcohol ($p<0.001$) and Champagne ($p<0.001$) intervention leading to significant reductions in mTOR levels relative to the isocaloric control (Figure 9). However, in a similar manner to that observed in the hippocampus, both alcohol and Champagne evoked significant increases in cortical phospho-CREB ($p<0.05$). There were no significant alterations in the activation state, or expression of, Akt, Arc, Bcl-xL, BDNF, CNPase, Erk, JNK, p38 and pro-BDNF in the cortex (Table 1).

DISCUSSION

The development of cognitive impairment and motor deficits during aging is a complex process that begins in middle age, even in the absence of symptomatic neurodegenerative disease (15). Human clinical trials and animal studies have identified flavonoid-rich foods and beverages as being capable of delaying the onset of age-related cognitive impairment (21, 22, 43, 51, 53, 65, 74). Flavonoids present in such foods have been postulated to evoke protection against cognitive aging through the actions of absorbed flavonoids and their metabolites on neurons and glia, enhancing existing neuronal function and/or to stimulating neuronal regeneration (21, 27, 51, 65). Although antioxidant mechanisms cannot be excluded (75), recent data sets suggest that such effects are mediated by their ability to modulate neuronal signalling (50, 52, 54), stimulate neurotransmitter release (23), increase cerebrovascular blood flow (14) and even stimulate hippocampal neurogenesis (5). Although a large body of evidence has accumulated for flavonoid-rich foods (27), there is less known regarding the influence of non-flavonoid phenolics on similar behavioral and molecular parameters. We have previously shown that such phenolics (caffeic acid, tyrosol, *p*-coumaric acid and chlorogenic acid) are capable of modulating neuronal signalling *in vitro* (36, 63), and enhance blood flow *in vivo*, suggesting that foods/beverages containing such components may also be capable of inducing cognitive improvements (67).

Despite the well-established harmful effects of heavy alcohol intake (19), epidemiological data suggest that moderate wine consumption may reduce the incidence of age-related dementia, including Alzheimer's disease (39, 40, 73). Animal data support these findings, indicating that a moderate consumption of red wine attenuates Aβ-neuropathology in a mouse model of Alzheimer's disease (70). In the current study we show that Champagne wine, which is rich in phenolics, such as gallic acid, protocatechuic acid, tyrosol, caftaric acid and caffeic acid, but essentially poor or free of flavonoids, is also capable of enhancing spatial working memory (without altering motor performance) in aged animals. In contrast, moderate alcohol intake failed to induce spatial memory changes. These observations are in agreement with those observed following long-term red wine intake in a similar model of hippocampal-dependent spatial memory (2).

The effects of Champagne on spatial memory were paralleled by a number of changes in hippocampal and cortical protein expression, which may underpin performance on spatial memory tasks. Perhaps most

notably, dystrophin and CNPase were modulated by Champagne supplementation but not alcohol. CNPase (2',3'-cyclic nucleotide 3'-phos phodiesterase) is a myelin-associated enzyme that constitutes around 4% of total CNS myelin protein, and is thought to undergo significant age-associated changes (17), and is reduced in Alzheimer's disease and Down's syndrome patients (69). Furthermore, Champagne-induced hippocampal increases in the cytoskeletal associated protein, dystrophin, may be beneficial as a lack of this protein in the hippocampus has been associated with impaired cognitive function (37), spatial memory (61) and long-term potentiation (60). Indeed, patients lacking dystrophin in the hippocampus and neocortex (due to mutation in the dystrophin gene) display a range of cognitive deficits (1). Intervention with the phenolic rich Champagne also led to the increased expression of a range of 'other' cytoskeletal proteins, including plakoglobin (γ-catenin), spectrin, calponin, cytokeratin pep4 and pep19, myosin Va and Focal Adhesion Kinase. Such proteins facilitate complex neuronsal network formation in the brain and operate with neuronal membrane proteins (e.g., ion channels, scaffolding proteins, and adaptor proteins) at sites of synaptic contacts to regulate synaptogenesis and coordinate synaptic strength (16, 41).

BDNF, a major regulator of synaptic transmission and plasticity at adult synapses (32) has previously been shown to partly mediate flavonoid-induced improvement in spatial memory through the ERK/Akt-CREB-BDNF pathway (44, 74). We show that phenolics present in Champagne also increase BDNF and CREB levels in the hippocampus and a number of other neuroplastic proteins, notably dystrophin, plakoglobin, tryptophane hydroxylase, CNPase. CREB is well known to be involved in memory and learning processes (55), via its selective activation of numerous downstream genes including BDNF (3) and the CREB/BDNF pathway plays a critical role in synaptic plasticity and memory consolidation (35). In contrast to previous findings with flavonoid-rich interventions, we observed no significant modulation of ERK1/2 or Akt by Champagne intervention, suggesting that BDNF is not regulated in this manner. Rather phenolic-induced BDNF expression may be regulated through p38 signalling in the hippocampus, leading to a decrease in the ERK/p38 activity ratio and the direct inhibition of raf-1 by p38 (18). Indeed, raf-1 was significantly down-regulated by Champagne intervention, together with a number of other proteins connected to raf-1 in the hippocampus (p38, ATF2 , GADD 153, p57kip2, Cdc27, Cyclin A, E2F1, PCAF, c-myc, GRB2, FAK and spectrin) and in the cortex (p38, Cyclin A, Cyclin D1, c-myc, caspase 7, NGRF p75, PTEN, Phospholipase C g1 and A2 group V, PKC a b and g, NMDAR 2a, Pyk2 and Tau), as indicated by STRING functional

protein association networks (20). Furthermore, the observed increases in p38 activation are in agreement with previous findings, showing that p38 activity may mediate BDNF synthesis in the rat hippocampus (24).

Furthermore, unlike flavonoids, Champagne phenolics did not specifically regulate Akt-mTOR signalling, although alcohol treatment did appear to increase in the activation status of mTOR, a protein that regulates survival, differentiation and development of neurons and is crucial for synaptic plasticity, learning and memory formation in adult brain (56), something which agrees with previous datasets (30). Similarly, reductions in hippocampal levels of Bcl-xL levels were reduced by both Champagne and alcohol, suggesting that alcohol intake itself may play a role in apoptotic regulation in the brain (13). A number of other apoptotic proteins were found to be significantly down-regulated by champagne in the hippocampus (Bcl-xL, Bcl-x, caspases 5, 6 and 11, PSR and GADD 153,) and in the cortex (Bcl-xL, Bcl-x, Bcl-10, caspases 7 and 11), although it is unclear whether alcohol alone would also mediate similar reductions. Anti-apoptotic proteins such as Bcl-xL and other members of the Bcl-2 family have been implicated in the selective neuronal loss observed in AD (49) and have been found to be up-regulated in Alzheimer brains (25).

In summary, the potential of Champagne to beneficially influence spatial memory appears to be mediated by its ability to induce a number of changes in hippocampal and cortical protein expression, which occur in parallel to behavioral effects. Such effects on protein expression may result via the direct actions of individual phenolics such as tyrosol, caffeic acid and their metabolites or their synergistic effect on hippocampal and cortical neurons/glia following their uptake into the brain. Although we did not observe this directly, previous data support the potential for such phenolics to undergo transfer across the blood brain barrier (BBB) (11, 46). Whilst the ability of flavonoids to cross the BBB is believed to be dependent on lipophilicity, small phenolics may transverse the BBB via amino acid transporters, such as has been reported for 4-ethylcatechol (34). Furthermore, caffeic acid shares structural similarities with L-DOPA and, as such, may undergo BBB transport via catecholamine transporter systems. Together, our data indicate that moderate Champagne intake, and phenolic acid intake in general, may be beneficial in counteracting memory impairments associated with aging, a finding that broadly supports human epidemiological data (26, 28, 29, 39).

INNOVATION

Flavonoid-rich foods and beverages have been shown to exert a multiplicity of neuroprotective effects in the brain, whereas little is known regarding the actions of smaller phenolics/polyphenols. Our data provide the first evidence that phenolics such as gallic acid, protocatechuic acid, tyrosol, caftaric acid and caffeic acid, delivered via moderate Champagne wine intake, also induce improvements in spatial memory in aged rodents and are dependent on the potential of these phenolics to modulate the expression of a number of proteins in the cortex and hippocampus related to the control of cell signaling, neuronal plasticity, cell cycle and apoptosis.

MATERIALS AND METHODS

Materials

Gallic acid, protocatechuic acid, caffeic acid, Bradford reagent, panorama antibody array Cell Signaling Kit, CelLytic™ MT Cell Lysis Reagent and HRP-conjugated goat anti-rabbit and anti-mouse secondary antibodies were obtained from Sigma (Poole, UK). Caftaric acid was obtained from Apin Chemicals (Abingdon, UK). Tyrosol was purchased from Extrasynthese (Lyon, France). PhosSTOP phosphatase inhibitors and cOmplete Mini protease inhibitors were obtained from Roche (Burgess Hill, UK). Precision Plus Protein Western C Standards and Precision Protein StrepTactin-HRP Conjugate were purchased from Biorad (Hemel Hempstead, UK). Primary antibodies used were: anti-BDNF (1:200) and anti-pro-BDNF (1:200) from Millipore (Watford, UK); anti-phospho-p38 Thr180/Tyr182 (1:1000), anti-total-p38 (1:1000), anti-phospho-CREB Ser133 (1:1000), anti-total-CREB (1:1000), anti-phospho-Erk1/2 Thr202/Tyr204 (1:2000), anti-total-Erk1/2 (1:2000), anti-phospho-Akt Ser473 (1:1000), anti-total-Akt (1:1000), anti-phospho-JNK Thr183/Tyr185 (1:1000), anti-total-JNK (1:1000), anti-Arc/Arg 3.1 C-7 (1:1000), anti-phospho-mTOR Ser2448 (1:1000), and anti-mTOR (1:1000) from New England Biolabs (Hitchin, UK); anti-phospho-PKA C-α (1:1000), anti-total-PKA C-α (1:1000), anti CNPase (1:1000), anti-dystrophin (1:500) and anti-Bcl-xL (1:500) from Sigma (Poole, UK). ECL reagent, and Hyperfilm-ECL were purchased from Amersham Biosciences (Amersham, UK). All other reagents were obtained from Sigma (Poole, UK) or Merck (Nottingham, UK).

Extraction and quantification of polyphenols from Champagne Wine.

Champagne extracts were prepared using Champagne wine derived from both Chardonnay and Pinot Noir and Pinot Meunier grapes as reported previously (68). Briefly, 375ml of Champagne were degassed, filtered, concentrated under vacuum and extracted with ethyl acetate. The organic phase was separated from the aqueous phase and concentrated under reduced pressure to yield a brown aromatic residue (yield=1g/375ml). The obtained fraction was dissolved in methanol, filtered and analyzed by HPLC. HPLC analysis was carried out with an Agilent 1100 series liquid chromatograph equipped with a diode array

detector linked to the HP ChemStation Software system. Samples were analyzed by reverse-phase HPLC using a Nova-Pak C18 column (4.6 × 250mm, 4 µm particle size) fitted with a guard column C18 NovaPak from Waters (Elstree, UK). The temperature of the column was maintained at 30°C. The mobile phases consisted of a mixture of aqueous methanol 5% in 0.1% hydrochloric acid 5M (A) and a mixture of aqueous acetonitrile 50% in 0.1% hydrochloric acid 5M (B) and were pumped through the column at 0.7 ml/min. The following gradient system was used (min/% B): 0/5, 5/5, 40/50, 55/100, 59,9/100, 60/5, with 10 min post-run. The eluent was monitored by photodiode array detection at 280 and 320 nm, and spectra of products were obtained over the 200–600 nm range. Calibration curves of the phenolic compounds were constructed using authentic standards (0.1–100 mg/ml) and in each case were found to be linear with correlation coefficients of .0.995. All data were analyzed using ChemStation software.

Animals and supplementation: All procedures were conducted according to the specifications of the United Kingdom Animals (Scientific Procedures) Act, 1986 and to the national guidelines for the care and use of animals. Three groups of adult, male Wistar rats (n = 8 per group; housed in cages each containing 2 rats; Harlan, UK) were kept, under controlled temperature (22±1°C) and humidity (50%±10%), and on a 12:12 light-dark cycle (lights off 10:00 hours). All rats were 15 months old at the beginning of the trial. Animals were pseudo-randomly assigned to 3 groups based on their baseline spatial working memory correct choice scores. Once allocated to a group, animals were fed daily with either Champagne, an iso-caloric control drink or an iso-caloric drink containing alcohol (Figure 1D). The iso-caloric control was matched for macro- and micronutrient-content, notably glycerol, fructose, glucose and citric acid content and was dissolved in carbonated water. The alcohol control was similarly matched but also contained alcohol (12.5% vol). The Champagne wine used in the study also contained phenolic compounds (gallic acid, protocatechuic acid, tyrosol, caftaric acid, caffeic acid). To control daily intake of the intervention diets, the three drinks were prepared freshly each day and administered in the form of a mash, by mixing the test solutions with a small amount (8g/10ml) of powdered standard diet (RM3, Special Diets Services, UK), and the correct amount given to each rat based on body weight. The level of Champagne and alcohol supplementation was approximately equivalent to a glass per day for human consumption (1.78 ml/kg bw). At the end of the spatial memory trials animals were sacrificed and brains removed, the hippocampal and cortical regions dissected, snap-frozen in liquid nitrogen, and stored at – 80 °C until analysis.

Spatial working memory: Spatial working memory performance was assessed using a cross-maze apparatus (38), which is a commonly used paradigm to evaluate spatial learning, working memory and reference memory performance in rodents (48, 74). All rats were fasted overnight before the procedure to provide sufficient motivation to ensure a high level of response during testing. Extra-maze cues (laboratory furniture, lights, and several prominent visual features on the walls) were held constant throughout the experiment. Rats were given 2 weeks pre-training (habituation) in order that they ran reliably from the start arm to locate reward pellets in both goal arms of the maze. This was followed by 6 weeks of shaping sessions (each consisting of six trials) prior to initiation of the supplementation regimes. During each shaping trial, rats were trained to enter an open goal arm and collect a reward pellet (entry to the alternate other goal arm was restricted). Across each shaping session, the open goal arm varied between trials according to a pseudorandom design. Following completion of the shaping sessions, dietary supplementation and testing sessions were started. During the testing phase, each animal received 8 trials per test session, with each consisting of a sample phase and a choice phase. During the sample phase, rats were placed in the start arm and allowed access only one goal arm to recover a food reward. Once the reward pellet had been collected, animals were removed and returned to the start arm for 10 s. During the choice phase, both goal arms were accessible and animals were allowed a free choice between the two, with correct choice responses recorded for animals entering arm not visited during the sample phase. An animal was deemed to have selected an arm when it had placed a hind foot within the confines of the arm correct arm (retracing was measured as a failure on the task). Correct choices were rewarded with a food reward, whereas incorrect arm choices meant the animal was returned to its home cage. For 50% of the choice phases the rat was placed in the “alternate” start arm opposite to the normal start arm. For each trial, accuracy and time taken to make a choice were measured. The maze was cleaned with 50% ethanol solution between trials to remove any olfactory clues. Each test session contained a pseudo-random sequence of correct choices between the two arms, as well as a pseudo-random sequence for the start arm during the choice phase. Rats were given 2 test sessions on the cross-maze, one session administered immediately before supplementation was started (baseline) and one administered at 6 weeks. Each group

of aged animals was pseudo-randomly assigned before the intervention based on baseline correct choices scores.

Stationary beam test: The motor skills of the animals during champagne supplementation were tested on a stationary beam (57) at baseline and at the end of the intervention (6 weeks). The dynamic equilibrium was tested on a beam consisting of a scale (150 cm, 3cm diameter) located 40 cm above a soft carpet. At the beginning of the experiment, the rat was placed on the middle of the beam, its body axis perpendicular to the beam's longitudinal axis. Distance covered and latency times before falling were recorded, and the walking speed was calculated. The trial was stopped when the animal fell or 3 mins had elapsed.

Antibody array: The Panorama Antibody array Cell Signalling Kit (Sigma-Aldrich) was used, which contains 224 different antibodies spotted in duplicate on nitrocellulose glass slides, and represents proteins involved in biological pathways such as apoptosis, cell cycle, nuclear proteins, neurobiology, cytoskeleton and signal transduction. Three biological replicates were used for hippocampus region and two biological replicates for cortex region. Statistical analysis was conducted on biological replicates and significantly modulated protein expression in the hippocampus and in the cortex was reported in table 1 and table 2 respectively. Proteins from both brain regions of champagne-treated and control-treated animals were extracted, purified, labeled with Cy-fluorescent dyes, hybridized to microarrays (at concentration of 5 μg/ml of each labeled target) and washed according to the protocols provided by the manufacturer. Briefly, protein extracts were prepared using the extraction buffer provided in the kit, and protein concentration was determined in the supernatant by the Bradford method. 1 mg of protein extract from each sample was labelled with Cy5 or Cy3, according to the manufacturer's instructions. Free, non-incorporated Cy3 and Cy5 dyes were separated by applying the labeled extracts on SigmaSpin Post-Reaction Clean-Up Columns. The dye to protein molar ratio (D/P) was calculated and only samples having D/P > 2 were used. An equal amount of labeled protein of both extracts (5μg/ml) was incubated on the Microarray slide for 30 min; all washes were done in PBS-Tween 0.05%. Microarray slides were scanned on GenePix 4200 Professional

scanner (Molecular Devices), with laser power reduced to 25% and PMT settings manually adjusted to avoid saturated pixels in the experimental spots, and resulted images were analyzed with GenePixPro 6 software (Molecular Devices). Spot intensities were local background adjusted, and spots with negative values were removed from further analysis. For remaining spots, resulted intensities were averaged for replicates, treatment-to-control ratios calculated, log2-transformed and normalized to median.

Western immunoblotting: Dissected brain regions were homogenized on ice with a glass homogenizer using CelLytic™ MT Cell Lysis Reagent (Sigma, UK) containing cOmplete Mini protease inhibitors and PhosSTOP phosphatase inhibitors. Homogenates were left on ice for 45 min before centrifugation at 4000 g for 5 min at 4 °C to remove unbroken cell debris and nuclei. Protein concentration in the supernatants was determined by the Bradford protein assay. Samples were incubated for 2 min at 95 °C in boiling buffer (final concentration 62.5 mM Tris, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.0025% bromophenol blue). Boiled samples (20–60 µg/lane) and protein standards were run on 9–12% SDS-polyacrylamide gels and proteins were transferred to nitrocellulose membranes (Hybond-ECL; Amersham) by semidry electroblotting (1.5 mA/cm2). The nitrocellulose membrane was then incubated in a blocking buffer (20 mM Tris, pH 7.5, 150 mM NaCl; TBS) containing 4% (w/v) skimmed milk powder for 45 min at room temperature followed by 2 × 5 min washes in TBS supplemented with 0.05% (v/v) Tween 20 (TTBS). Blots were then incubated with the primary antibodies of interest (1:200 - 1:2000 dilution) in TTBS containing 1% (w/v) skimmed milk powder (antibody buffer) overnight at 4°C on a three-dimensional rocking table. The blots were washed 2 × 10 min in TTBS and incubated with secondary anti-rabbit or anti-mouse IgG conjugated to HRP (1:1000 dilution) and Precision Protein StrepTactin-HRP Conjugate (1:10.000 dilution) for 45 min. Finally blots were washed 2 × 10 min in TTBS rinsed in TBS and exposed to ECL reagent for 1–2 min and developed. Bands were analyzed using Bio-Rad Quantity One 1-D Analysis software. Molecular weights of the bands were calculated from comparison with pre-stained molecular weight markers that were run in parallel with the samples. Relative band intensities were calculated as a ratio of the phosphorylated protein to total protein in the case of CREB, p38 and mTOR. For BDNF, dystrophin, CNPase and Bcl-xL, relative band intensity was calculated in comparison to GAPDH levels.

Statistics: All behavioral data (means ± SEM, n=8) were subjected to two-way analysis of variance for repeated measures with diet group (control, alcohol and champagne) and time (0, 6 weeks) as main factors, followed by a Bonferroni post-hoc test to examine differences between individual treatments (GraphPad Prism 5). For the microarray data (means ± SEM, n=3 for hippocampus, n=2 for cortex), to evaluate the difference in protein expression between champagne-treated and iso-caloric control-treated samples, we used Rank Product Test (4), as implemented in MeV 4.6 microarray data analysis software (45), with cut-off of log2-ratio more than 0.4 or less then -0.4 (more than 1.3-fold change) and $P<0.05$. For the western immunoblotting data, (means ± SEM, n≥3) the statistical evaluation of the results was performed by one-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls post-hoc t-test (GraphPad Prism 5). Significant changes are indicated as follows: a = $P< 0.001$; b = $P< 0.01$; c = $P< 0.05$. Correlations between animal performance on spatial memory tasks and protein levels in the brain were calculated using the Pearson product moment correlation coefficient and the associated P value.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

LIST OF ABBREVIATIONS

Akt = Akt/Protein kinase B

Arc = Activity-regulated cytoskeleton-associated protein

ATF2 = Activating Transcription Factor 2

BBB = Blood Brain Barrier

Bcl-x = B-cell lymphoma 2–associated x protein

Bcl-xL = B-cell lymphoma2-extra large protein

BDNF = Brain-Derived Neurotrophic Factor

Cdc27 = Cell division cycle protein 27

c-myc = Myc proto-oncogene protein

CNPase = 2',3'-cyclic-nucleotide 3'-phosphodiesterase

CNS = Central Nervous System

COX-2 = Cyclooxygenase-2

CREB = cAMP response element-binding protein

E2F1 = Transcription factor E2F1

Erk = Extracellular-signal-regulated kinase

FAK = Focal Adhesion Kinase

GADD 153 = Growth arrest and DNA damage-inducible protein 153

GRB2 = Growth factor receptor-bound protein 2

iNOS = Inducible Nitric Oxide Synthase

JNK = c-Jun Amino-terminal Kinases

L-DOPA = L-3,4-dihydroxyphenylalanine

MAPK = mitogen-activated protein kinase

MPTP = 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

mTOR = mammalian target of rapamycin

NGFR p75 = p75 Nerve Growth Factor Receptor, low affinity

NMDAR 2a = N-methyl-D-aspartate Receptor 2a

NO = Nitric Oxide

p38 = Mitogen-activated protein kinase 14 (p38 alpha)

p57kip2 = cyclin-dependent kinase inhibitor 1C, p57 isoform 1

PCAF = P300/CBP-associated factor

PGE2 = Prostaglandin E2

PKA C-α = cAMP-dependent protein kinase catalytic subunit alpha

PKC a = Protein kinase C alpha

PKC b = Protein kinase C beta

PKC g = Protein kinase C gamma

pro-BDNF = Brain-Derived Neurotrophic Factor precursor

PSR = Phosphatidylserine Receptor

PTEN = Phosphatase and tensin homolog

Pyk2 = Protein tyrosine kinase 2

raf-1 = RAF proto-oncogene serine/threonine-protein kinase

STRING = Search Tool for the Retrieval of Interacting Genes/Proteins

Tau = microtubule-associated protein tau

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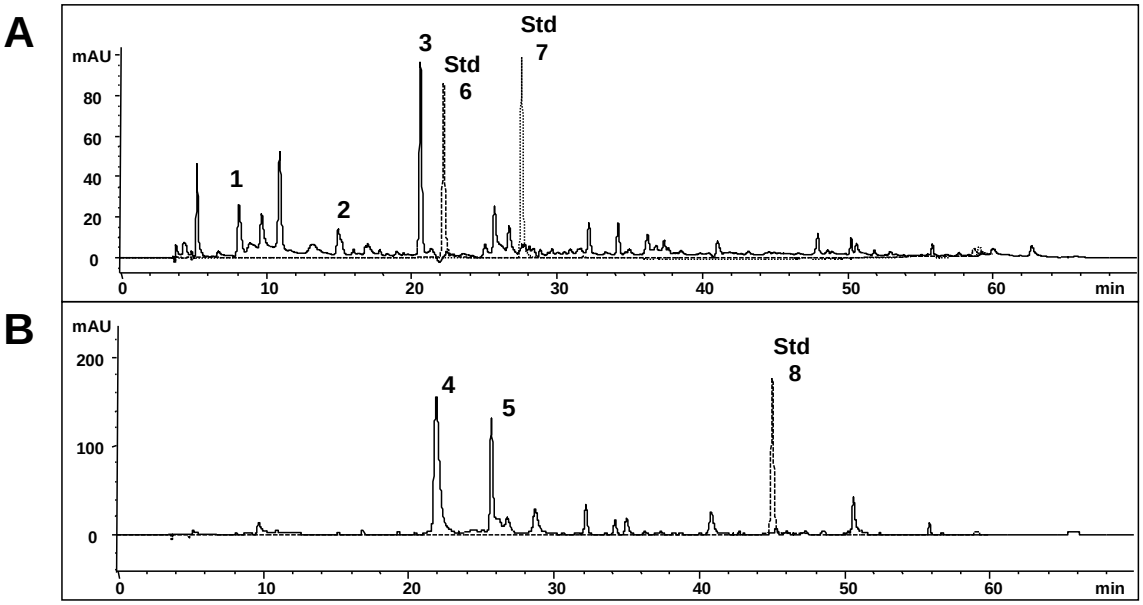
TABLES

Table 1. Immunoblotting analysis of proteins extracted from hippocampus and cortex

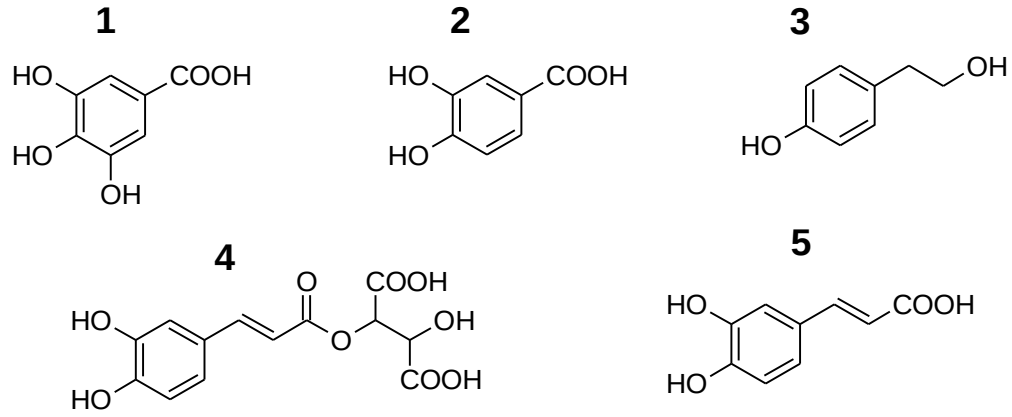
	Control		Champagne		Alcohol	
	mean	SEM	mean	SEM	mean	SEM
Hippocampus						
Akt ph/tot	0.581	0.023	0.535	0.009	0.543	0.004
Arc/GAPDH	0.722	0.130	0.684	0.214	0.803	0.059
Erk1 ph/tot	0.271	0.009	0.260	0.016	0.248	0.019
Erk2 ph/tot	0.116	0.003	0.120	0.013	0.101	0.008
JNK 1 ph/tot	0.949	0.064	1.043	0.111	0.853	0.010
JNK 2/3 ph/tot	0.672	0.035	0.666	0.057	0.598	0.043
PKA C-α ph/tot	0.022	0.015	0.017	0.008	0.044	0.015
pro-BDNF/GAPDH	0.372	0.050	0.517	0.048	0.284	0.081
Cortex						
Akt ph/tot	0.782	0.011	0.734	0.024	0.732	0.025
Arc/GAPDH	0.577	0.050	0.648	0.010	0.598	0.028
Bcl-xL/GAPDH	0.536	0.081	0.680	0.077	0.693	0.042
BDNF/GAPDH	1.000	0.034	0.995	0.075	0.803	0.089
CNPase/GAPDH ratio	1.413	0.144	1.915	0.183	1.662	0.064
Erk1 ph/tot	0.955	0.177	0.731	0.043	0.845	0.041
Erk2 ph/tot	0.682	0.120	0.586	0.032	0.673	0.024
JNK 1 ph/tot	0.880	0.014	0.917	0.013	0.921	0.012
JNK 2/3 ph/tot	0.757	0.030	0.744	0.107	0.757	0.013
p38 ph/tot	0.732	0.077	0.903	0.002	0.593	0.086
pro-BDNF/GAPDH	0.500	0.032	0.586	0.050	0.302	0.109

Statistical analysis (One-way ANOVA) was performed. Differences were all non-significant ($p>0.05$).

Figure 1



C



D

Analysis of constituents in the three drinks

Constituents	Iso-caloric control drink	Iso-caloric alcohol drink	Champagne wine
phenolic compounds			
gallic acid (mg/L)	-	-	0.66
protocatechuic acid (mg/L)	-	-	0.50
tyrosol (mg/L)	-	-	8.46
caftaric acid (mg/L)	-	-	5.01
caffeic acid (mg/L)	-	-	1.43
total phenolics (mg/L)	-	-	16.06
other constituents			
Fructose (g/L)	3.50	3.50	3.50
Glucose (g/L)	3.00	3.00	3.00
Glycerol (g/L)	6.20	6.20	6.20
Citric acid (g/L)	0.02	0.02	0.02
alcohol (% vol)	-	12.50	12.50

Figure 2

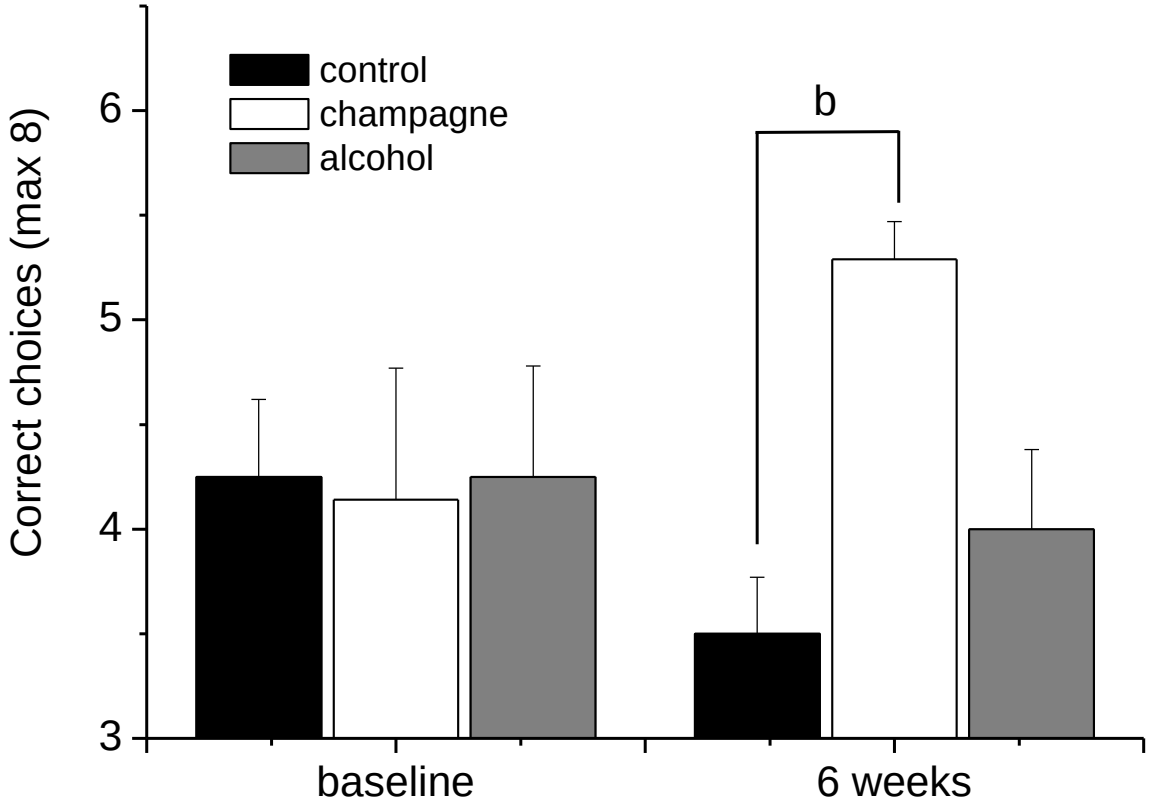

control
champagne
alcohol
b
Correct choices (max 8)
6
5
4
3
baseline
6 weeks

Figure 3

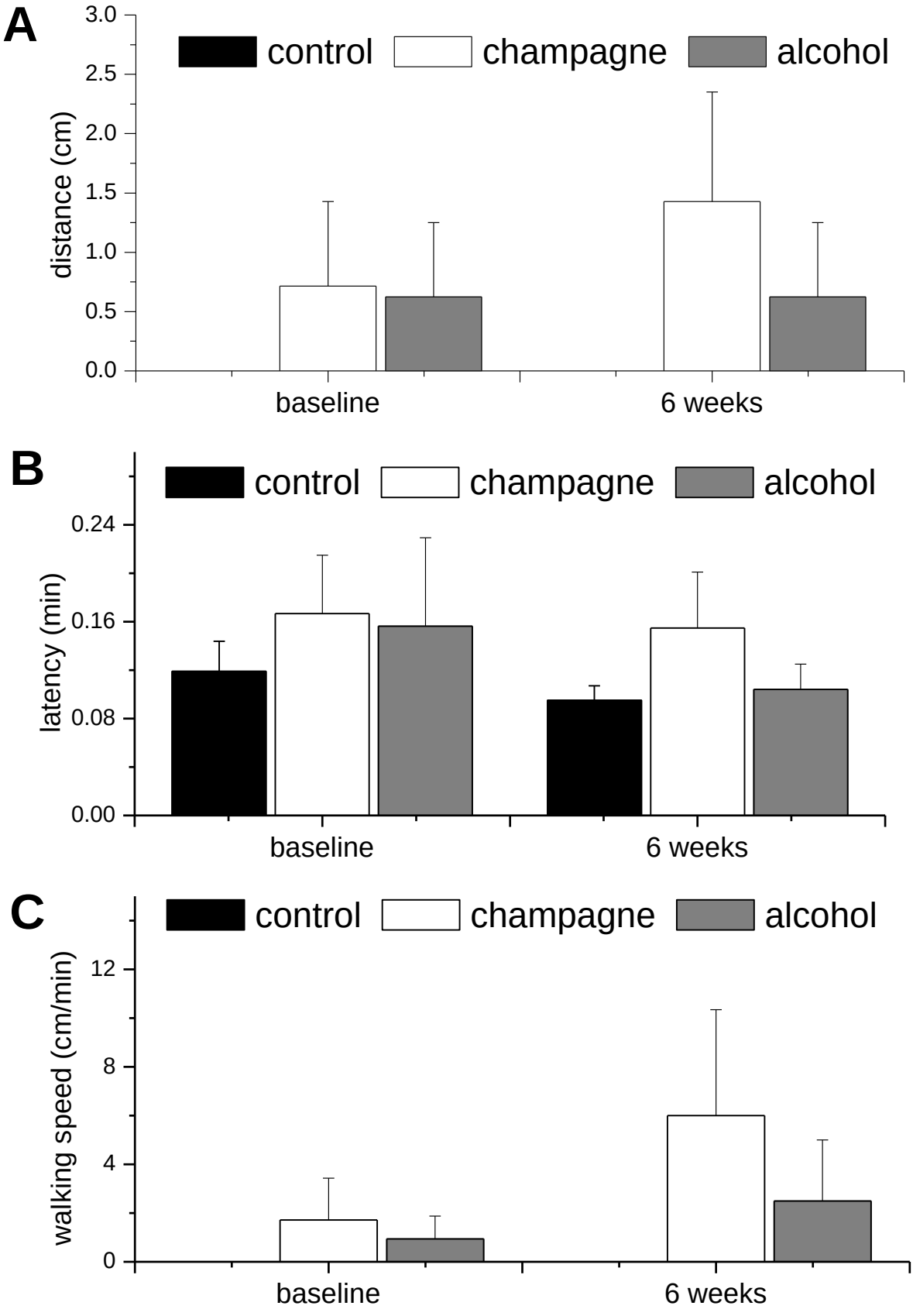

A
control
champagne
alcohol
distance (cm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
baseline
6 weeks
B
control
champagne
alcohol
latency (min)
0.24
0.16
0.08
0.00
baseline
6 weeks
C
control
champagne
alcohol
walking speed (cm/min)
12
8
4
0
baseline
6 weeks

Figure 4

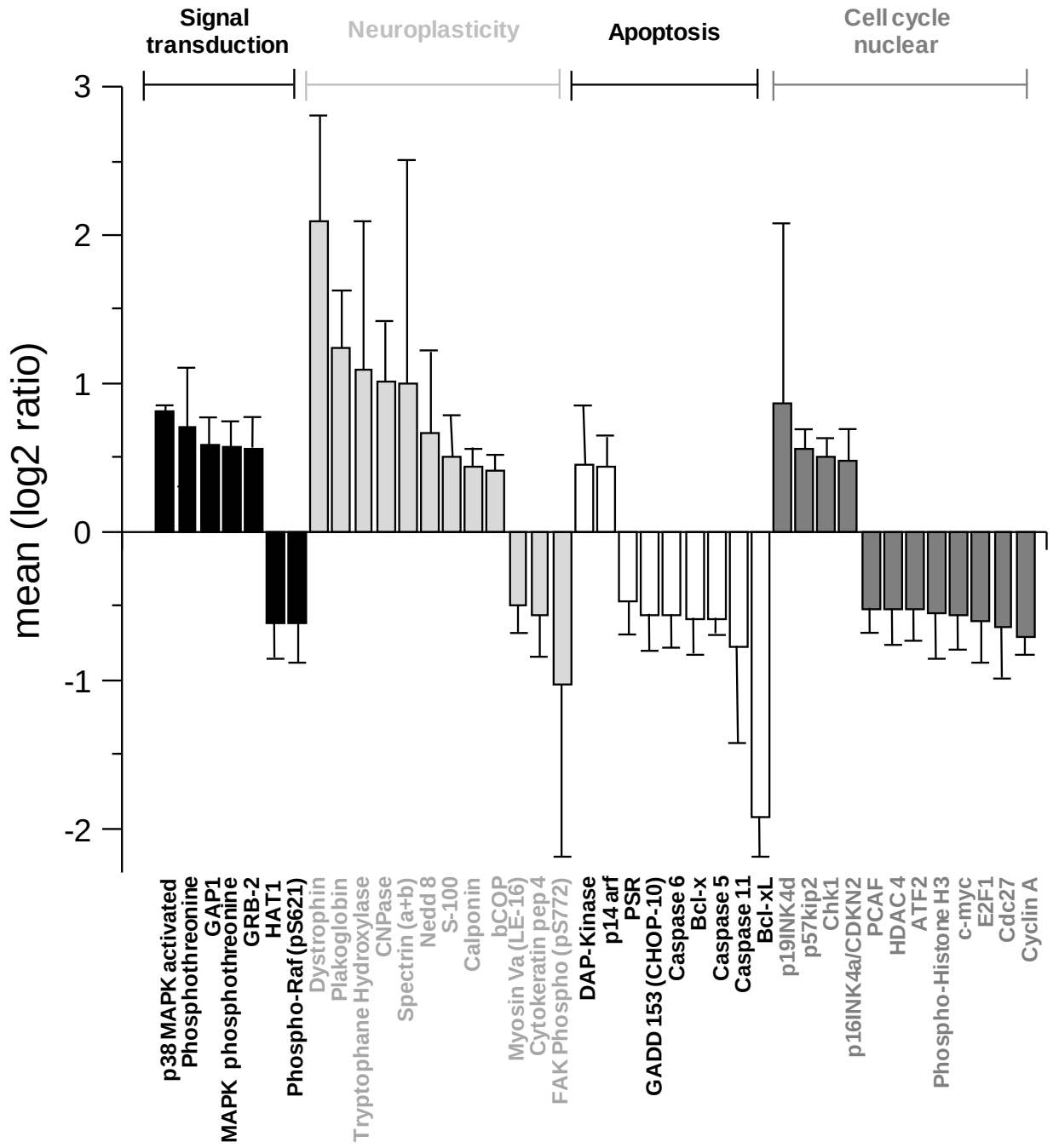

Signal transduction
Neuroplasticity
Apoptosis
Cell cycle nuclear
mean (log2 ratio)
3
2
1
0
-1
-2
p38 MAPK activated
Phosphothreonine
GAP1
MAPK phosphothreonine
GRB-2
HAT1
Phospho-Raf (pS621)
Dystrophin
Plakoglobin
Tryptophane Hydroxylase
CNPase
Spectrin (a+b)
Nedd 8
S-100
Calponin
bCOP
Myosin Va (LE-16)
Cytokeratin pep 4
FAK Phospho (pS772)
DAP-Kinase
p14 arf
PSR
GADD 153 (CHOP-10)
Caspase 6
Bcl-x
Caspase 5
Caspase 11
Bcl-xL
p19INK4d
p57kip2
Chk1
p16INK4a/CDKN2
PCAF
HDAC4
ATF2
Phospho-Histone H3
c-myc
E2F1
Cdc27
Cyclin A

Figure 5

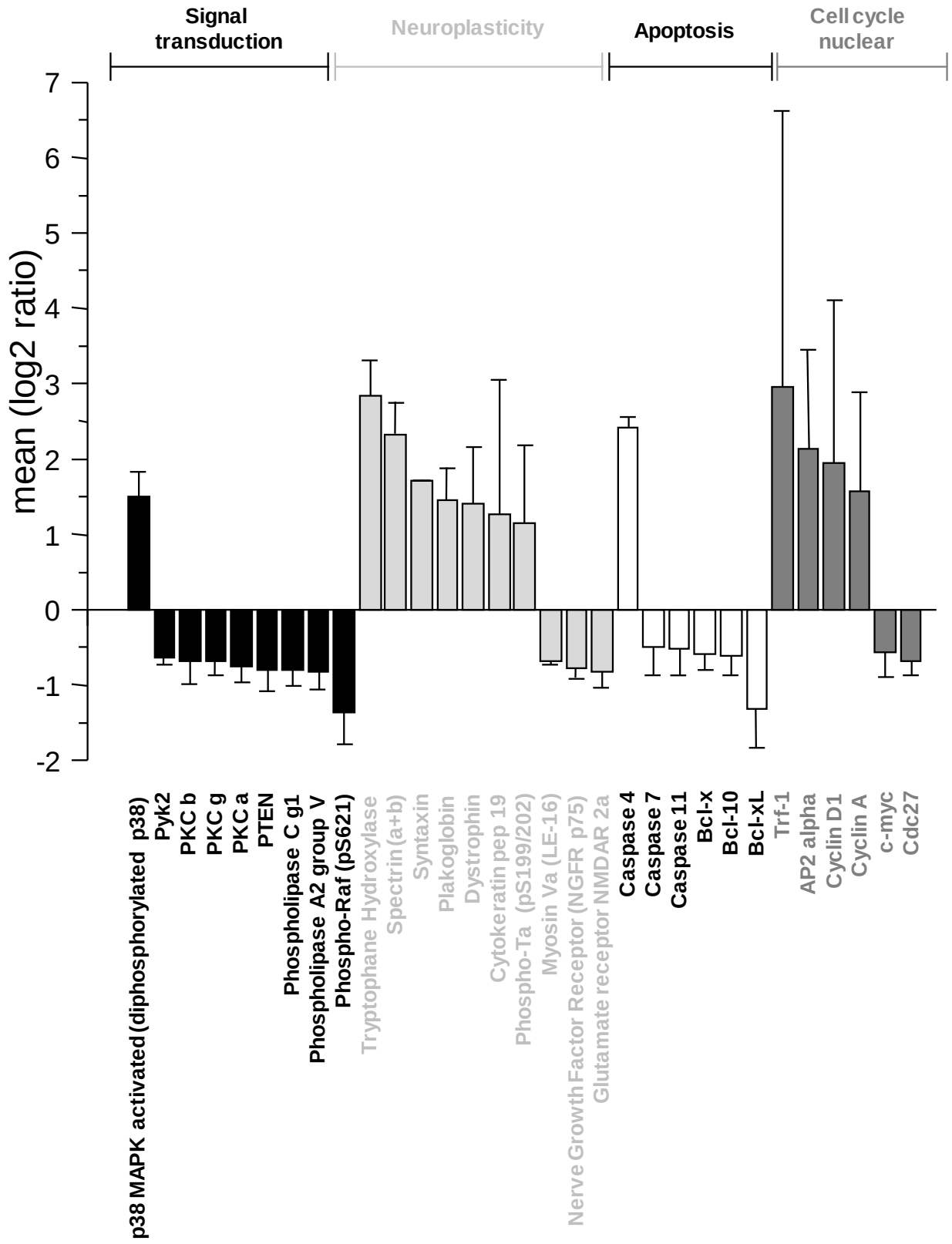

Signal transduction
Neuroplasticity
Apoptosis
Cell cycle nuclear
mean (log2 ratio)
7
6
5
4
3
2
1
0
-1
-2
p38 MAPK activated (diphosphorylated p38)
Pyk2
PKC b
PKC g
PKC a
PTEN
Phospholipase C g1
Phospholipase A2 group V
Phospho-Raf (pS621)
Tryptophane Hydroxylase
Spectrin (a+b)
Syntaxin
Plakoglobin
Dystrophin
Cytokeratin pep 19
Phospho-Ta (pS199/202)
Myosin Va (LE-16)
Nerve Growth Factor Receptor (NGFR p75)
Glutamate receptor NMDAR 2a
Caspase 4
Caspase 7
Caspase 11
Bcl-x
Bcl-10
Bcl-xL
Trf-1
AP2 alpha
Cyclin D1
Cyclin A
c-myc
Cdc27

Figure 6

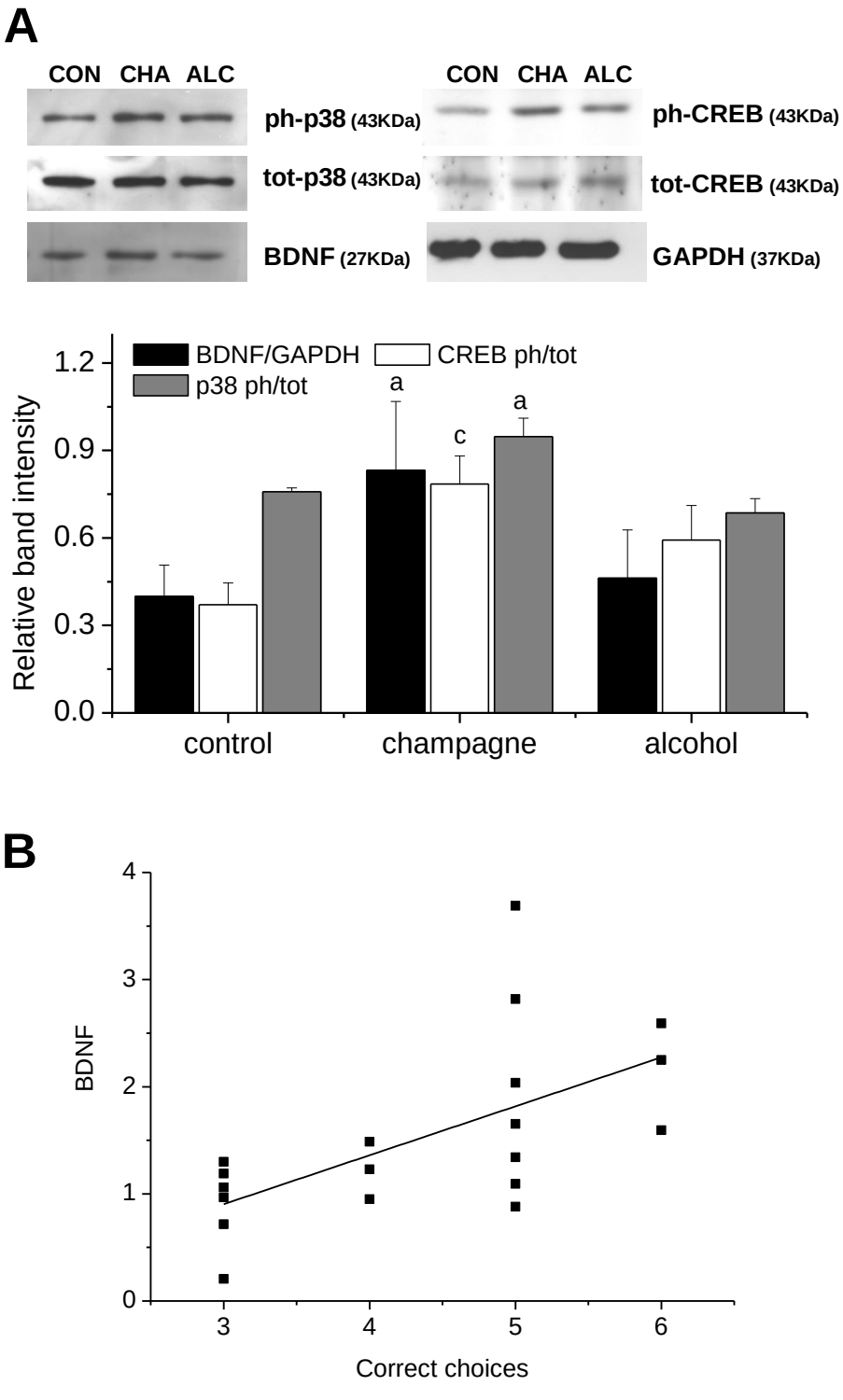

A
CON CHA ALC
CON CHA ALC
ph-p38 (43KDa)
ph-CREB (43KDa)
tot-p38 (43KDa)
tot-CREB (43KDa)
BDNF (27KDa)
GAPDH (37KDa)
BDNF/GAPDH
CREB ph/tot
p38 ph/tot
Relative band intensity
1.2
0.9
0.6
0.3
0.0
a
c
a
control
champagne
alcohol
B
BDNF
4
3
2
1
0
3
4
5
6
Correct choices

Figure 7

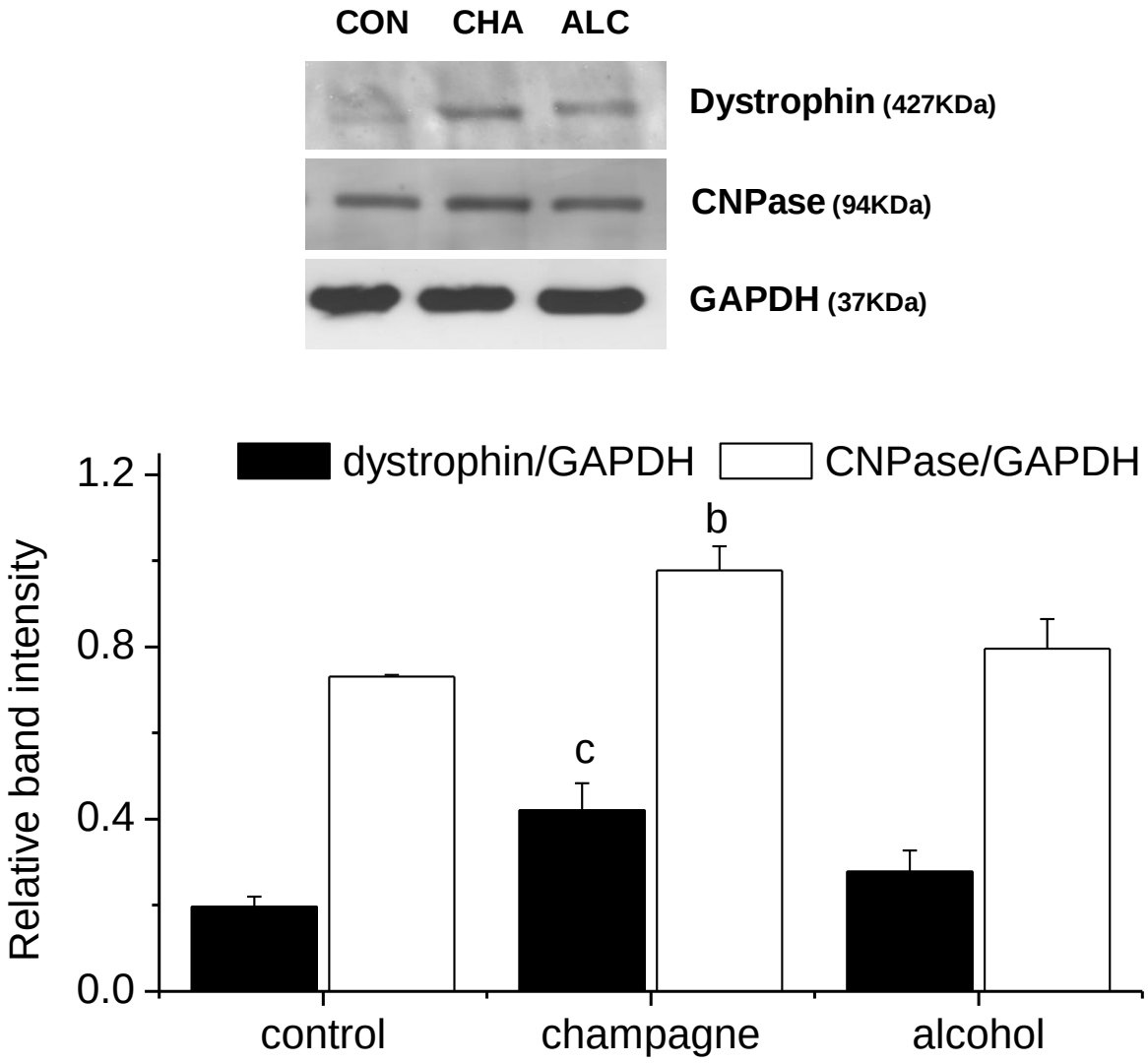
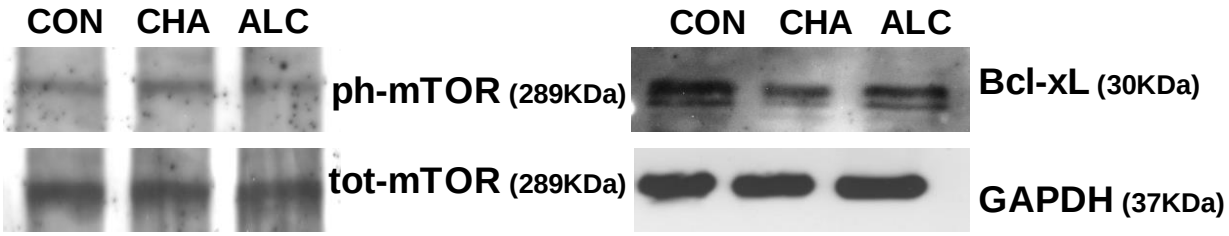

CON
CHA
ALC
Dystrophin (427KDa)
CNPase (94KDa)
GAPDH (37KDa)
dystrophin/GAPDH
CNPase/GAPDH
Relative band intensity
1.2
0.8
0.4
0.0
b
c
control
champagne
alcohol

Figure 8


CON
CHA
ALC
ph-mTOR (289KDa)
tot-mTOR (289KDa)
CON
CHA
ALC
Bcl-xL (30KDa)
GAPDH (37KDa)

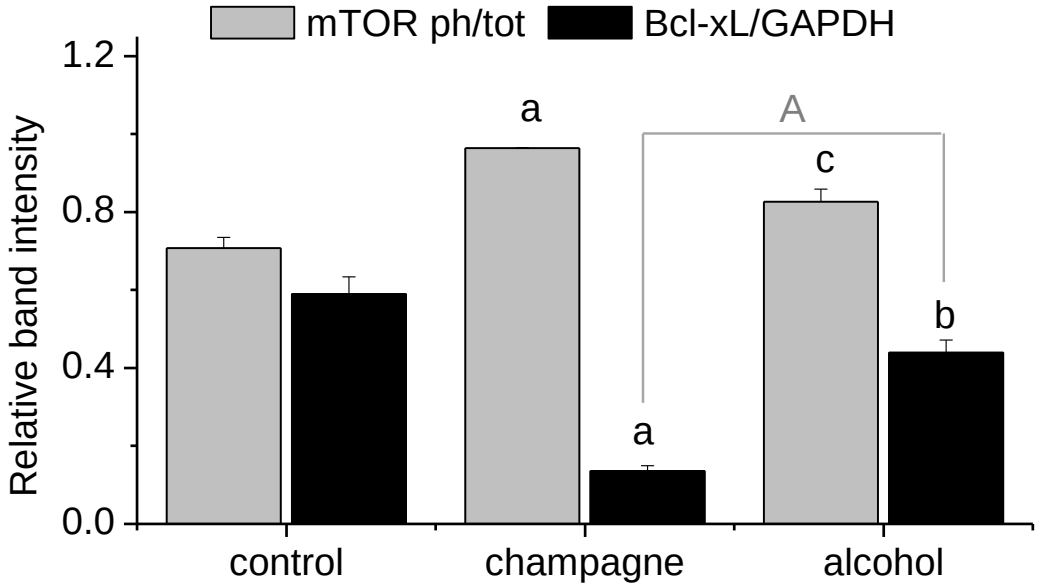

mTOR ph/tot
Bcl-xL/GAPDH
Relative band intensity
1.2
0.8
0.4
0.0
a
a
A
c
b
control
champagne
alcohol

Figure 9


CON
CHA
ALC
ph-mTOR (289KDa)
tot-mTOR (289KDa)
CON
CHA
ALC
ph-CREB (43KDa)
tot-CREB (43KDa)

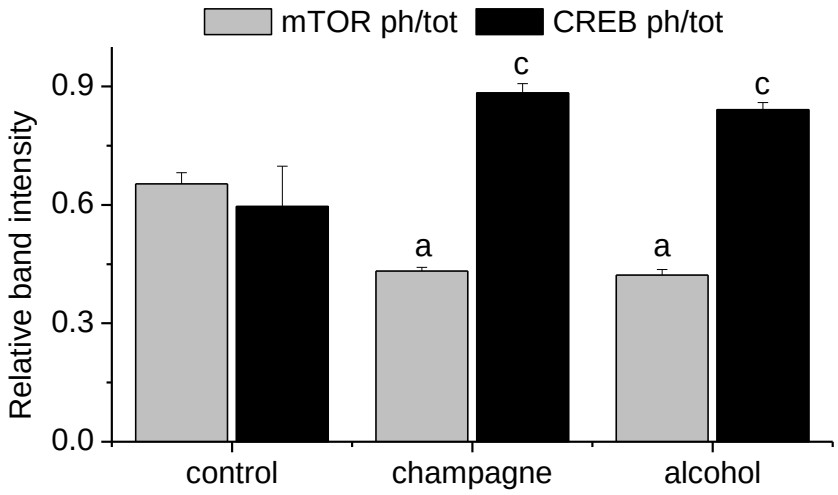

mTOR ph/tot
CREB ph/tot
Relative band intensity
0.0
0.3
0.6
0.9
a
a
c
c
control
champagne
alcohol

FIGURE LEGENDS

Figure 1. Major constituents of the control and test interventions. Chromatographic separation of the aqueous/organic extract of Champagne wine at: (A) 280nm and (B) 320nm indicating quantified phenolics in the extract (1 to 5) and flavonoid standards of epicatechin (6) catechin (7) and resveratrol (8) to point out their absence in the extract. (C): Structures of major phenolic compounds present: 1: gallic acid; 2: protocatechuic acid; 3: tyrosol; 4: caftaric acid; 5: caffeic acid. (D) Quantification of phenolic compounds and of other major constituents of the control and test interventions.

Figure 2. Effect of Champagne supplementation on rat's spatial memory performance measured as choice accuracy (number of correct choices) in a T maze alternation task. Maximum score is 8 correct choices. Results are presented as means ±SEM (n=8). Two-way ANOVA indicated a trend to significance on the interaction between treatment and time ($F_{3.536}=2.955$, $p=0.075$) and Bonferroni post-hoc test indicated a significant effect of Champagne vs control at 6 weeks (b = $P<0.01$).

Figure 3. Effect of Champagne supplementation on rat's motor skills (stationary beam scores) in champagne supplemented animals (A) Distance. (B) Latency time. (C) walking speed. Results are presented as means ±SEM (n=8). No significant differences were found (two-way ANOVA).

Figure 4. Differentially expressed proteins (antibody array) in the hippocampus between Champagne and control treated animals. Results are presented as mean log2-ratios ±SD (n=4). All changes were statistically significant ($p<0.05$).

Figure 5. Differentially expressed proteins (antibody array) in the cortex between champagne and control treated animals. Results are presented as mean log2-ratios ±SD (n=4). All changes were statistically significant ($p<0.05$).

Figure 6. Immunoblotting analysis of proteins extracted from hippocampus. (A) Crude lysates (20-40μg protein) were immunoblotted with antibodies that specifically recognize phospho-p38, total-p38, phospho-CREB, total-CREB, BDNF, and GAPDH. Results (phospho/total ratio for p38 and CREB; protein/GAPDH

ratio for BDNF) are presented as means ± SEM (n ≥ 6). a = p < 0.001; c = p < 0.05 represent significant differences vs control (One-way ANOVA). (B) Correlation between choice accuracy in spatial memory task and hippocampal BDNF protein levels, after 6 weeks of dietary interventions, (n=19; r=0.613; P<0.01).

Figure 7. Immunoblotting analysis of proteins extracted from hippocampus. Crude lysates (20-40μg protein) were immunoblotted with antibodies that specifically recognize dystrophin, CNPase and GAPDH. Results (protein/GAPDH ratios) are presented as means ± SEM (n ≥ 6). b = p < 0.01; c = p < 0.05 represent significant differences vs control (One-way ANOVA).

Figure 8. Immunoblotting analysis of proteins extracted from hippocampus. Crude lysates (20-40μg protein) were immunoblotted with antibodies that specifically recognize phospho-mTOR, total-mTOR, Bcl-xL and GAPDH. Results (phospho/total ratio for mTOR; protein/GAPDH ratio for Bcl-xL) are presented as means ± SEM (n ≥ 3). a = p < 0.001; b = p < 0.01; c = p < 0.05 represent significant differences vs control. A = p < 0.001 represent significant differences vs alcohol (One-way ANOVA).

Figure 9. Immunoblotting analysis of proteins extracted from cortex. (A) Crude cortical lysates (20-40μg protein) were immunoblotted with antibodies that specifically recognize phospho-mTOR, total-mTOR, phospho-CREB and total CREB. Results (phospho/total ratio for mTOR; protein/GAPDH ratio for CREB) are presented as means ± SEM (n ≥ 3). a = p < 0.001; c = p < 0.05 represent significant differences vs. control (One-way ANOVA).